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Escherichia coli O antigen typing using DNA microarrays☆

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Abstract

DNA microarrays were developed for rapid identification of different serogroups of *Escherichia coli* in a single platform. Oligonucleotides, as well as PCR products from genes in the O antigen gene clusters of *E. coli* serogroups O7, O104, O111, and O157 were spotted onto glass slides. This was followed by hybridization with labeled long PCR products of the entire O antigen gene clusters of these serogroups. Results demonstrated that microarrays consisting of either oligonucleotides or PCR products generated specific signals for each serogroup. This is the first report describing the development of model DNA microarrays for determining the serogroup of *E. coli* strains.

Keywords: DNA microarray; Serotyping; *Escherichia coli*; O7; O104; O111; O157

1. Introduction

Escherichia coli serotyping is conventionally performed by agglutination reactions using antisera raised in rabbits against the ca. 179 different O standard references strains. However, traditional serotyping is laborious and time consuming, and often generates equivocal results due to cross-reactions among different serogroups. Furthermore, the antisera for serotyping can only be generated by specialized laboratories with animal facilities. Therefore, rapid molecular methods for identifying different *E. coli* serogroups are needed.

The O antigen, which contains many repeats of an oligosaccharide unit (O unit), is present in the outer membrane of Gram-negative bacteria and contributes to the antigenic variability of the cell surface. The genes involved in the biosynthesis of O antigens in *E. coli* are located in the O antigen gene cluster flanked by the *galF* and *gnd* genes on the *E. coli* chromosome. A number of *E. coli* O antigen gene clusters have been sequenced, and the genes were annotated [3,11,12,18,25–27]. Several genes in the clusters, in particular the *wzx* (O antigen flippase) and *wzy* (O antigen polymerase) genes show relatively low similarity among different *E. coli* serogroups, and PCR primers targeting the *wzx* and *wzy* genes have been used to develop serogroup-specific PCR assays [3,11,12,18,25–27]. However, since the primers are each specific for the respective O antigen gene cluster genes, PCR assays targeting each of the serogroups need to be performed to identify the *E. coli* strain. Therefore, an assay for parallel detection of multiple serogroups in a single platform is needed.

DNA microarray technology has emerged in recent years as a very a powerful tool for simultaneous detection of large numbers of DNA sequences in a sample. Discrete zones of DNA targets less than 1 mm in diameter (spots) are immobilized to a planar surface and exposed to a sample containing labeled DNA fragments. Complementary fragments in the fluorescent dye-labeled sample hybridize to the target spots, and unbound or weakly bound fragments are removed through washing. The amount of hybridized DNA on each spot is measured using a fluorescence scanner. Microarray technology has been increasingly applied in the field of drug discovery [10], clinical diagnostics [24] and toxicogenomics [14]. While most microarray applications have focused on gene expression analysis, applications of DNA microarrays for the detection and identification of bacteria and viruses are being expanded [13]. DNA microarrays possess considerable advantages and potential for development into rapid and sensitive methods for the detection and characterization of pathogenic

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microorganisms. For example, multiple microorganisms can be detected simultaneously, and the presence of virulence genes and antibiotic resistance genes can also be detected. Recently, various types of microarrays have been developed and evaluated for pathogen detection in food and environmental samples and for biodefense applications [1,15,22]. Genetic markers used for microarray-based microbial detection include housekeeping genes such as 16S-23S rDNA [7], *gyrB* genes [16] and virulence genes [9]. Microarray techniques have been used to distinguish not only different bacterial genera [28], but also different strains within the same species such as *Listeria monocytogenes* [4,5] and different *Salmonella* serovars [2]. Although direct detection and discrimination of nucleic acids from bacteria is feasible, there has been no report on the use of microarrays for typing *E. coli* based on the O antigen serogroups.

Our objective was to develop model DNA microarrays to distinguish different *E. coli* serogroups. The *E. coli* O types O7, O104, O111, and O157 were chosen for this pilot study since strains belonging to these four serogroups are human pathogens [8,19,21,23], and the availability of the O antigen gene cluster sequences of these serogroups made it possible to design unique DNA targets for each serogroup [18,25–27].

2. Material and methods

2.1. DNA oligonucleotide array design and fabrication

The four bacterial strains, *E. coli* O7 Bi 7509–41, *E. coli* O104 H 519, *E. coli* O111 Stoke W, and *E. coli* O157 A2 were from the collection described by Ørskov et al. [20] and are used as standard strains for serotyping. The sequences of the O antigen gene clusters for *E. coli* serotypes O7, O111, O104, and O157 were used to select the target sequences characteristic to each serogroup. The GenBank accession numbers were as follows: AF125322 (O7), AF078736 (O111), AF361371 (O104), and AF061251 (O157). The OligoArraySelector program [6] was used to select unique oligonucleotides (35-mers) for each *E. coli* serogroup (Table 1). The specificity of oligonucleotides selected by the OligoArraySelector software was further determined using the NCBI blastn program against the non-redundant (nr) database, and analyses revealed that the oligonucleotides showed homology only to the corresponding *E. coli* serogroup. Four, nine, seven, and five oligonucleotides were selected from the *E. coli* O7, O104, O111, and O157 O antigen gene cluster sequences, respectively. The oligonucleotides were synthesized with amino linkers at the 5′-termini (Biosearch Technologies, Inc.) for covalent attachment to superaldehyde coated slides (Telechem, ArrayIt, Sunnyvale, CA). Oligonucleotides were spotted at a concentration of 50 μM in 1× MicroPrinting Plus solution (Telechem) using a SpotBot Personal Microarrayer (Telechem) according to the manufacturer's instructions. Each oligonucleotide was spotted in triplicate using SMP4 pins (Telechem). The distance between the spots was 750 μm, and the spot diameter was ~135 μm. The printed slides were dried at room temperature overnight. Unbound oligonucleotides were removed by washing the slides with 0.2% SDS for 2 min and 2×SSC consisting of 0.3 M NaCl/0.03 M trisodium citrate, pH 7.2 for

Table 1
List of oligonucleotides used for microarray analysis

Name	Target	Sequence	Tm (°C)	Positions on the array
AF125322O7_13492	*E. coli* O7	TTATTAGATTGGACGATAAAATATTTCGGAACTGT	68	2A
AF125322O7_9684		AGTTATAAACAACAGTGAATTATTGATTGGCGATT	69	3B
AF125322O7_7558		ATAATGTAAGGATTGATGATTTTTGCGTGTTTTCA	71	4B
AF125322O7_6746		AATAGCTGATAATTATGGATTGAAAGTAATCTACG	65	5B
AF361371O104_10820	*E. coli* O104	GAAATAATTTCCTCCGCTCATTTACTATTGTAATA	66	4C
AF361371O104_9825		AACCTTTCTTGCAATAGTTCTTTCCTTTATATTGA	68	4D
AF361371O104_8830		AATTATTACAATTTTTGTGACCATTGCTTCTCAAG	69	5C
AF361371O104_8318		AAATATGTTCATTTCTTAGGTTTTCAGACTAATCC	66	5D
AF361371O104_7332		ATCGCAACAAAGTAGAAATAACTACTCATATCAAT	66	6C
AF361371O104_7253		CAAAAGCCTTAAAAAGGAATACAGTTATAATCTTC	66	6D
AF361371O104_6347		TTATTTGCAATTATCTTTGCTTACGTTCATGAAAG	70	7C
AF361371O104_4701		TTATTTTATGGTTGTTTTTCATCCTGAAACGCTTA	70	7D
AF361371O104_3836		GTAATTATCTACTTTTTATCATTGCCTCCAGTTAA	65	1E
AF078736O111_11820	*E. coli* O111	GTTTCATTGTACGCAAAAAGAATAATATATCCTGT	67	2E
AF078736O111_10952		TATTATATGAGAGAAATGCTCCTTTCATTGTTGTA	67	2F
AF078736O111_10116		ATGTTTGTTAATGCGGAGGATCTATTTAGATAAAA	68	3E
AF078736O111_7754		TCACTAATAAACCAAAACTTAAAAGAGGTGATGAA	68	3F
AF078736O111_7384		GTTATACTGGGAGTTTTTCTCATGATTTAACAAAA	67	4E
AF078736O111_7319		AATGCTGGTTTAGGTTACGATTATTCAATTAATGA	68	4F
AF078736O111_5985		ATACTACATTGTTGGTTTATTAGCGGAAGTTTTTT	67	5E
AF061251O157_14131	*E. coli* O157	AATACTCTGATACATTTTTATACGTTATTCAAGCC	65	9B
AF061251O157_13595		AACCAATAACTATAAAACATCCAAATAGCGTTGTT	68	10A
AF061251O157_6675		ATCTTTTAATACAGAACGCATAGACCATATTTATC	65	11A
AF061251O157_5764		TAAGCGAGAAATAGATCAAAGGAATGTGTTTTTTA	69	12A
AF061251O157_5612		TAAGAGCTCTGCCTTTTATTCATATTTTGATAGTA	66	12B

Table 2
Primers used for amplification of the *wzx* and *wzy* genes of *E. coli* O7, O104, O111, and O157

Oligonucleotide primer set	Base position and accession numbers	Target genes	Expected size of PCR product (bp)
O7wzx-4938F	4938–5715	*wzx*	778
O7wzx-5715R	AF125322		
O104wzx-3633F	9099-9630	*wzx*	532
O104wzx-3634R	AF361371		
O104wzy-3629F	6493–6952	*wzy*	460
O104wzy-3630R	AF361371		
O104wzy-3580F	6308–7319	*wzy*	1012
O104wzy-3581R	AF361371		
O111wzx-969F	8646–9908	*wzx*	1263
O111wzx-970R	AF078736		
O111wzx-1061F	9150–9754	*wzx*	605
O111wzx-1063R	AF078736		
O111wzx-1060F	8906–9468	*wzx*	563
O111wzx-1062R	AF078736		
O111wzy-900F	9976–10827	*wzy*	852
O111wzy-901R	AF078736		
O111wzy-980F	10113–10484	*wzy*	372
O111wzy-983R	AF078736		
O157wzy-839F	839–1051	*wzy*	213
O157wzy-1051R	AF061251		
O157wzy-1034F	1034–1276	*wzy*	243
O157wzy-1276R	AF061251		

2 min, followed by boiling in water for 2–5 min. The slides were then fixed in cold ethanol for 3 min and dried for 1 min using a microarray high-speed centrifuge (Telechem).

2.2. Probe labeling and hybridization

Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Long PCR assays were performed to amplify the O antigen gene cluster using the Expand Long Template PCR system (Roche Applied Science, Mannheim, Germany) and JUMPSTART (named for just upstream of many polysaccharide-associated gene STARTs) and GND (6-phosphogluconate dehydrogenase) primers that flank the *E. coli* O antigen gene clusters [27]. The sequence of the JUMPSTART sense primer was 5′-ATTGGTAGCTGTAAGCCAAGGGCGGTAGCGT-3′, and the antisense GND primer sequence was 5′-CACTGCCATACC-GACGACGCCGATCTGTTGCTTGG-3′ (Invitrogen Life Technologies, Inc., Carlsbad, CA). The long PCR conditions were as described previously [12]. The long PCR products were verified on 0.8% agarose gels and purified according to instructions in the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA). Purified long PCR products were labeled with 5-(3-aminoallyl)-dUTP according to instructions in the BioPrime Array CGH Genomic Labeling System (Invitrogen Life Technologies, Inc., Carlsbad, CA), followed by the fluorescent dye Alexa Fluor 546 using the ARES™ DNA labeling kit (Molecular Probes, Inc., Eugene, OR). The labeled probes were purified using the Purification Module from the BioPrime Array CGH Genomic Labeling System (Invitrogen Life Technologies, Inc.). Purified probes were dissolved in 3 μl of dH_2O, boiled for 3 min and chilled on ice. Twelve microliters of 1.25×UniHyb buffer (Telechem) were added to the denatured probe, and the hybridization mixtures were prewarmed at 65 °C for 10 min before hybridizing onto the glass slides containing the oligonucleotides. The hybridizations were performed in hybridization cassettes (Telechem) either at 42 °C for 1 or 5 h, or overnight. After hybridization, the slides were washed in 2×SSC/0.2% SDS at 42 °C for 2 min, and 2×SSC at room temperature for 2 min, followed by drying for 1 min using a microarray high-speed centrifuge. The slides were scanned using a Tecan LS400 Scanner (Tecan, Research Trangle Park, NC) at 20 μm, PMT 200. All hybridization experiments were repeated three times.

2.3. PCR array fabrication and hybridization

Since PCR primers specific for the *E. coli* O antigen gene cluster *wzx* (O antigen flippase) and *wzy* (O antigen polymerase) genes have been used previously for PCR assays [18,25–27], the possibility of spotting PCR products of the *wzx* and *wzy* genes of the different *E. coli* serogroups rather than oligonucleotides on the slides was investigated. PCR primers targeting the *wzx* and *wzy* genes of *E. coli* O7, O104, O111, and O157 that have been used successfully in PCR assays [18,25–27] are listed in Table 2. The sizes of the PCR products ranged from 213 to 1263 bp (Table 2). One primer set for *E. coli* O7 (*wzx*), three for *E. coli* O104 (*wzx* and *wzy*), five for *E. coli* O111 (*wzx* and *wzy*), and two for *E. coli* O157 (*wzy*) were used (Table 2). The 50 μl PCR reaction contained 150 ng of template genomic DNA, 0.4 μM (each) of the primers (Integrated DNA Technologies, Inc.), 0.2 mM of each of four dNTPs (dTAP, dCTP, dGTP and dTTP), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ and 1.25 U of Taq DNA polymerase. PCR reactions were performed using a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, Foster City, CA) using the following conditions: (i) an initial denaturation step of 2 min at 94 °C; (ii) 30 cycles, with 1 cycle consisting of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, and a final extension of 10 min at 72 °C. Five microliters of the PCR products were visualized using agarose (1%) gel electrophoresis and staining with ethidium bromide. The PCR products of different sizes (Table 2) were purified using the QIAquick PCR Purification Kit (Qiagen, Inc.) and spotted onto superamine-coated slides (Telechem, ArrayIt, CA) using the SpotBot Personal Arrayer. After printing, the slides were baked at 80 °C, followed by UV cross-linking (UV Stratalinker 2400, Stratagene, La Jolla, CA) twice at 120 MJ. Slides with PCR products were processed, hybridized with the fluorescence-labeled (Alexa Fluor 546) long PCR products, washed, and scanned using the same conditions as with the oligonucleotide arrays.

3. Results and discussion

Fig. 1A demonstrates the layout of the oligonucleotide microarray slide showing the location of the specific

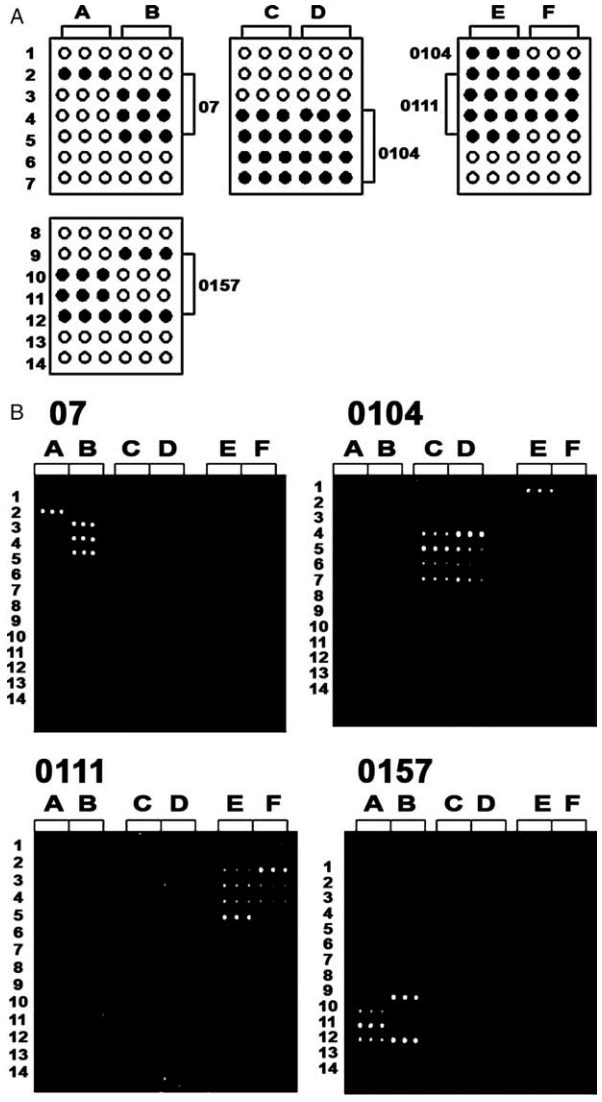

Fig. 1. (A) Location of oligonucleotides (bold spots) on the microarray slide. Oligonucleotides were spotted onto the array in triplicate. Oligonucleotides corresponding to the numbers and letters in the grids are indicated in Table 1. (B) Patterns of detection and differentiation among different *E. coli* serogroups (O7, O104, O111 and O157) using oligonucleotide-based DNA microarrays. Oligonucleotides were spotted onto the array in triplicate and hybridized to the long PCR products spanning the O antigen gene cluster of each serogroup. White dots represent the hybridization signals.

oligonucleotides (Table 1). Fig. 1B illustrates the hybridization patterns of *E. coli* O7, O104, O111 and O157. The four oligonucleotides specific for *E. coli* O7 (2A, 3B, 4B and 5B) were hybridized to the 15-kb long PCR product of the O antigen gene cluster of O7. Similarly, all of the nine oligonucleotides (4C-D, 5C-D, 6C-D, 7C-D, 1E) unique to *E. coli* O104 were hybridized to the O104 O antigen gene cluster long PCR product (12-kb). The long PCR products of the O antigen gene clusters of *E. coli* O111 (14-kb) and O157 (14-kb) also hybridized to the seven (2E-F, 3E-F, 4E-F and 5E) and five oligonucleotides (9B, 10A, 11A, 12A-B) characteristic to *E. coli* O111 and O157, respectively. The hybridization signals remained the same when different hybridization times (1', 5 h, and overnight) were used. In conclusion, the hybridization results clearly demonstrated that the oligonucleotide-based microarray accurately identified *E. coli* O7, O104, O111, and O157.

Fig. 2 demonstrates the hybridization patterns of *E. coli* O7, O104, O111, and O157 using PCR products as targets on the slide. As shown in Fig. 2, the PCR product of the *wzx* gene of *E. coli* O7 hybridized to the long PCR product of *E. coli* O7. Two PCR products from the *wzy* gene of *E. coli* O157 hybridized to the long PCR product of *E. coli* O157. Similarly, five and three PCR products of the *wzx* and *wzy* genes of *E. coli* O111 and O104, respectively, hybridized to the corresponding long PCR products of *E. coli* O111 and O104. No non-specific hybridization signals were observed. Fig. 2 clearly demonstrates that the PCR products of the *wzx* and *wzy* genes can be used in microarrays to identify and discriminate *E. coli* O7, O104, O111, and O157.

The current study represents the first report demonstrating that DNA microarrays using either oligonucleotides or PCR products as targets are feasible for identifying *E. coli* serogroups. Long PCR products over 10-kb were used for probe labeling, while in other studies in which microarrays were used for pathogen detection, short PCR products less than 1 kb were labeled [9]. There are some technical difficulties with labeling of long DNA fragments, and restriction enzyme digestion or sonication of the DNA is generally required. This is the first report demonstrating that long DNA fragments can be labeled with good efficiency without the need for digesting into smaller pieces. In the current study, *E. coli* genomic DNA was also labeled with fluorescence and hybridized to the arrays containing oligonucleotides and PCR products. Non-specific hybridization signals were occasionally obtained using this approach (data not shown). The non-specific hybridization signals may have resulted from the fact that short oligonucleotides (35-mers) may contain DNA sequences that are homologous to other regions of the genomic DNA, in addition to the specific regions in the O antigen gene clusters. Indeed,

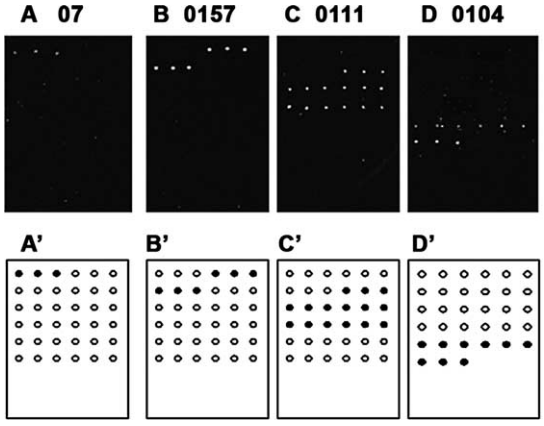

Fig. 2. Patterns of detection and differentiation among different *E. coli* serogroups (O7, O104, O111 and O157) using PCR product-based DNA microarrays. The PCR products were spotted on the array in triplicate and hybridized to the long PCR products of the O antigen gene cluster of each serogroup. (A–D) represent the microarray images of *E. coli* O7, O157, O111, and O104, respectively, and (A′–D′) represent the schematic array layout with the corresponding spots for each serotype in bold.

cross-hybridization has been reported to occur if the labeled DNA showed >75–80% homology to the oligonucleotide targets [17].

Generally, there are two types of targets that can be placed onto microarray glass slides: oligonucleotides and PCR products. No significant difference in sensitivity was observed between arrays containing oligonucleotides and PCR products [17]. Oligonucleotide arrays are costly to prepare but offer good specificity, while PCR arrays are less expensive, but can have less specificity. Compared with oligonucleotide arrays, PCR arrays were less useful for microbial detection due to low specificity [15]. In our study, both types of arrays were tested, and both generated acceptable results, offering the flexibility to choose different arrays based on different research needs. Our study shows that if carefully designed PCR arrays can also be useful for bacterial typing/identification.

Many O antigen gene clusters have been sequenced by us and other groups [3,11,12,18,25–27], and targets specific for other O antigen gene clusters can be selected and placed onto the typing array. There is the potential to expand the DNA array to contain target sequences/PCR products for all of the *E. coli* serogroups. We are in the process of analyzing the O antigen gene cluster sequences of other *E. coli* serogroups and placing serogroup-specific oligonucleotides and PCR products onto the array, since the results described in the current study have demonstrated the feasibility of this approach. In addition to the O antigen gene clusters, other genes including virulence genes that are unique to each serogroup can also be selected for microarray analysis. Identification of pathogen-specific markers with detection on DNA microarrays might allow more sensitive and rapid detection of pathogens than classical culture- or immunological-based methods and can also potentially be applied to the development of accurate identification and subtyping methods for use in food, biodefense, and environmental samples.

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